Oct 21,
1991

AFLATOXIN ELIMINATION WORKSHOP

Aflatoxin is recognized as a serious food safety hazard by most countries of the world. Producing food free of aflatoxin today requires a truly national effort and, particularly, the cooperation of both government and industry. The Agriculture Research Service and the commodity groups representing peanuts, corn, cottonseed and tree nuts recognize the importance of a strong national research effort to eliminate aflatoxin as a food safety threat.

This Aflatoxin Elimination Workshop, held in Atlanta, Georgia, is the fourth such yearly meeting held to review the ARS supported aflatoxin research and provide a forum for interested scientists to come together to discuss common problems and their potential solutions among themselves and with members of the industry. Thus although many of these scientists are performing very fundamental studies they gain a very clear idea of where their research is leading and the impact it will have on society. Also, this workshop provides the opportunity for gains in cost effectiveness of research by the recognition of common approaches and by sharing relevant information across commodities. This workshop has come to be recognized as the premier national meeting for advances leading to methods to eliminate aflatoxin.

Most of the research is performed by the ARS, however an important addition to this core effort is provided through a competitive award program provided by Congressional appropriations. This program is a unique effort of the ARS and representatives of the peanut, corn, cotton and tree nut industries. By extending the opportunity for the best university scientists to join the highly focused multithrust program, the rate of progress toward the elimination of aflatoxin is enchanced.

On the following pages are the abstracts of work presented at the 1991 Workshop.

Jane F. Robens
Agricultural Research Service
Workshop Program Coordinator

ECOLOGICAL AND PRODUCTION PARAMETERS

Ecology, Epidemiology, and Biocontrol of *Aspergillus flavus* and *A. parasiticus* in Pistachio Orchards in California.

Themis J. Michailides and **Mark A. Doster**, Dept. of Plant Pathology, Univ. of California, Berkeley / Kearney Ag. Center, Parlier, CA 93648; and **Noel F. Sommer**, Dept. of Pomology, Univ. of California, Davis, CA 95616.

Aspergillus flavus/parasiticus were found to develop on several types of debris (male and female inflorescences, fruit, leaves) from pistachio trees. Male inflorescences of pistachio trees may be an important substrate for *A. flavus/parasiticus* since they can be colonized by *Aspergillus* species throughout the summer. *A. flavus/parasiticus* developed from 11% of the male inflorescences gathered in early June and 27% of those gathered in late summer. Pistachio fruits that have fallen to the soil surface provide another substrate for development (fungi in the *A. flavus* group developed from 8.5% of these fruits). *A. flavus* grew and sporulated (although only slightly) in wounded pistachio leaves when inoculated in a pistachio orchard.

Our investigations confirmed that early split nuts (atypical pistachio fruits that have the hull and shell split revealing the kernel) and insect (navel orangeworm) damage increased infection of pistachio kernels by *A. flavus/parasiticus* in the orchard. In addition, early split nuts with shriveled hulls had more than twice the *A. flavus/parasiticus* infections than early splits with nonshriveled hulls. We noted that some commercial pistachio orchards had many fruits damaged by birds so that the kernel was exposed. *Aspergillus* species have been isolated from these bird damaged kernels.

Preliminary observations were made concerning the effect of cultural practices on the development of *A. flavus/parasiticus*. We used ten commercial pistachio orchards either having a cover crop or being disced regularly and having one of three types of irrigation: sprinkler, flood, or fanjet. *A. flavus* was isolated from every orchard. The orchards with a cover crop had more early split fruits than those orchards that were disced regularly (early split fruits are more likely to be infected by *A. flavus/parasiticus*). Also, more *A. niger* was isolated from soils from disced orchards than from orchards with cover crops (*A. flavus* was at levels too low to make a comparison). We observed abundant sporulation of *Aspergillus* species on debris (pistachio male inflorescences, pistachio leaves, and a weed, *Conyza* sp.) in two flood-irrigated orchards which suggests that flood irrigation may favor colonization of debris by *Aspergillus*.

In 1991 screening was continued for microorganisms that are antagonistic to *A. flavus*. Approximately 135 new isolates of microorganisms were tested. Preliminary testing of these potential antagonists were done using various media by evaluating the inhibition of *A. flavus* growth or the size of the inhibition zone between the antagonist and *A. flavus*. Eight of the most promising antagonists were further tested using pistachio fruits in the laboratory. All antagonist-treated fruits had less *A. flavus* than the control. A field test of four promising antagonists is now in progress.

Epidemiology of Preharvest Aflatoxin Contamination of Peanuts and Agronomic Practices to Prevent It

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Preharvest aflatoxin contamination of peanuts is the result of a complex interaction of many factors. The most important of these is drought stress during the pod maturation period, typically the last 30-60 days of the growing season. Without drought stress, significant preharvest aflatoxin contamination does not occur. However, when peanuts are subjected to drought stress during the latter part of the growing season, several other factors have an important influence on the extent to which contamination occurs.

Soil temperature is one of the more important factors involved in conjunction with drought stress. As a period of drought progresses, the soil temperature normally increases as more direct solar radiation reaches the soil surface through the receding canopy of peanut foliage. The drier, warmer soil environment is much more favorable for the aflatoxin-producing fungi, _Aspergillus flavus_ and _A. parasiticus_, and their populations in peanut soils under such conditions can increase dramatically. After an extended time in this environment the water activity of peanuts, another important factor, begins to decrease and it eventually reaches a point at which the phytoalexin-producing capacity of the peanut is lost. Without this important natural defense mechanism against fungal proliferation, the peanut is vulnerable to _A. flavus_ and _A. parasiticus_ as long as the kernel water activity remains high enough to support fungal growth and aflatoxin production. Although it is unclear why, pod maturity is also an important factor because immature peanuts are more susceptible to aflatoxin contamination than those that have reached a more advanced level of maturity by the time drought conditions are imposed. Finally, soil insects, particularly lesser cornstalk borer, aggravate the problem because of the avenues they provide for infection by _A. flavus_ and _A. parasiticus_.

Although many factors are involved in the epidemiology of aflatoxin contamination, the most significant is drought stress because of its direct affect on almost every other factor. Therefore, the agronomic practice that can have the greatest impact on aflatoxin contamination is adequate irrigation during the latter part of the growing season. There is no other known agronomic practice that can prevent aflatoxin contamination because the other factors involved in the process cannot be controlled with current agronomic practices.

However, late-season drought does not guarantee aflatoxin contamination if peanuts are harvested before contamination occurs. An aflatoxin early warning system has been shown to be an effective predictor of preharvest aflatoxin contamination when peanuts are subjected to late-season drought stress. Therefore, if adequate irrigation is not available to prevent drought from occurring, early warning systems could be utilized to alert the grower to harvest the peanuts earlier than normal, before significant contamination occurs.

Relationship of Lesser Cornstalk Borers to Aflatoxigenic Fungi in Peanuts

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Fungi that produce aflatoxins (*Aspergillus flavus* Link and *A. parasiticus* Speare) are known to invade peanuts during the growing season, and are favored when hot and dry conditions prevail. Thus, aflatoxin contamination in peanuts can be a serious problem in peanuts when the growing season is hot and dry. Lesser cornstalk borers (LCB) [*Elasmopalpus lignosellus* (Zeller)] are also favored by drought conditions and high temperatures. The larvae of these insects feed underground on peanut root hypocotyls, pegs and developing pods. In 1990, several collections of LCB larvae, and pegs and developing pods of plants, were taken from field plots at the Wiregrass Substation of the Alabama Agricultural Experiment Station in Southeast Alabama. Larvae were returned to the lab and evaluated for external and internal contamination with *A. flavus*-type fungi. Sixty-four percent of larvae were found to carry bits of the fungi on their cuticles, 29% were found to carry the fungi in their frass, and 9% of surface-sterilized larvae carried the fungus internally.

In 1990, drought conditions increased through the months of August and September. During that same time period, populations of LCB increased and incidence of *A. flavus*-type fungi increased on field-collected pegs, from 24% incidence on 15 August to 52% incidence on 13 September. Fungal incidence also increased on field-collected pods. Samples were taken at harvest for quality evaluations and individual pods were rated for insect damage. Evidence for insect damage was the presence of the insect itself or silken tubes from the LCB. Presence of aflatoxigenic fungi were also noted. Occurrence of insect damage in samples from plots that had never been treated with an insecticide was found to be 94% correlated to visible *A. flavus*-type fungi. This relationship changed when insecticides had been applied. In a study on the time of application of the insecticide chlorpyrifos, all treated plots had lower levels of insect damage and visible aflatoxigenic fungi than the untreated plots. Samples from insecticide-treated plots also had lower levels of aflatoxins, higher quality, higher yields and higher value. These data suggest the need for more in-depth studies relative to insecticide applications aimed at control of LCB that will minimize aflatoxigenic fungal invasion of pre-harvest peanuts.

In 1991 studies were continued, however, the moisture and temperature conditions were such that no LCB were found in field plots. Incidence of *A. flavus*-type fungi, over both years, was found to be positively correlated to number of days with a maximum temperature greater than 95°F. "LCB Days", previously developed for predicting population outbreaks of LCB, were also found to be positively correlated to incidence of aflatoxigenic fungi in field-collected pegs and pods. Studies will continue on establishing the relationship between these two organisms and developing methods of predicting problems due to these organisms.

1991 Aflatoxin Elimination Workshop
October 21, 1991

Status Report

Title: Pre-Harvest Monitoring and Control of Aflatoxin Formation in Cottonseed

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Abstract: This study focuses on obtaining information on the effect of various environmental factors on pre-harvest formation of aflatoxin in cottonseed and potential increases in aflatoxin levels during storage. Meteorological data, i.e., temperature, relative humidity, wind speed/direction, and precipitation, will be obtained using Arizona Meteorological Network (AZMET) stations located throughout cotton growing regions. Areas under study include the Yuma Valley, North Phoenix, Maricopa/Casa Grande, and Safford Valley. *Aspergillus* species spore load will be monitored on the cotton plant by collecting leaves and using modified Rose Bengal Agar medium for spore recovery/enumeration. Aflatoxin formation in cottonseed will be monitored during the growing and picking seasons by randomly collecting and testing cotton bolls in the field and from modules and trailers during harvesting. Aflatoxin contamination in cottonseed associated with individual fields will be followed through the ginning and feeding/crushing operations. Data obtained in this study will be evaluated to determine potential correlations between meteorological data and *Aspergillus* species spore load, and aflatoxin formation in the field as well as aflatoxin formation/increase during storage. This information can then be used to predict potential aflatoxin problems and subsequently, reducing the incidence and severity of aflatoxin contamination in cottonseed.

Agronomic practices that minimize aflatoxin contamination of cottonseed in the arid southwest.

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Aflatoxin contamination of cottonseed occurs at low levels sporadically across the cottonbelt. However, in the western desert valleys of Arizona and southern California contamination is perennially a serious concern. Contamination is also high some years in South Texas. Because contamination is most frequent and severe in western Arizona, we initiated field plots in the Yuma Valley in 1987 to investigate how we might alter agronomic practice to reduce contamination. Our results indicate that aflatoxin contamination of cottonseed in Arizona occurs in at least two phases. The crop is first contaminated with aflatoxins when Aspergillus flavus infects bolls damaged by pink bollworms (PBW) or other means, prior to maturity. This first phase is associated with BGYF on lint and it causes most of the contamination in the western desert valleys in some years. In our studies all highly contaminated bolls (bolls containing over 10,000 PPB) were PBW damaged and over 90 percent of aflatoxins detected occurred in PBW damaged bolls. All damaged bolls are not equally susceptible to contamination. Early bolls are more susceptible than bolls formed late in the season. This agrees with previous observations that most toxin occurs in bolls low on the plant. Based on these observations, protection of the first half of the crop from PBW and other boll damage is advised to reduce contamination.

A second contamination phase occurs when mature bolls are exposed to high humidity or rewetting at or after opening. Increases in toxin concentrations associated with this second phase can occur both prior to harvest and after harvest as the crop is held in modules and seed piles. Timely harvest should decrease the severity of both phases by reducing aflatoxin increases in early bolls after maturation and by reducing overwintering PBW populations and subsequent PBW damage to the early bolls of the next crop. To improve management of aflatoxin contamination, increasingly earlier harvest is recommended.

Postharvest handling of seed cotton and cottonseed can be very inconsistent. A third recommendation for limiting contamination is to harvest the crop dry and handle the crop properly. This applies to all handlers of the crop because at harvest the crop often has a very high level of Aspergillus flavus on the lint and seed. This fungal inoculum stays with the crop until it is either crushed or injested. Proper care is required to prevent the fungus from further infecting and contaminating the seed. These procedures include proper module construction and tarping, proper handling of ginned seed and proper storage of seed at gins, mills and by animal producers.

It is also suggested that a plan be devised to provide incentives to growers to produce cottonseed without aflatoxins. Growers have the greatest impact on the aflatoxin content of the crop. However, incentives to growers are generally inadequate.

10-21-91

Drought stress and epidemiology of *Aspergillus flavus* in corn.

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In 1991 we continued isolations of *Aspergillus flavus* populations from soil, cob residue, corn insects, air and grain from the Illinois River Valley Sand Field, Kilbourne, IL. Direct plating of 1/2 g soil in Botran medium at 37 C enabled us to detect *A. flavus* populations in 12/40 two gram soil samples (Ave. 0.45 CFU's/g soil) from the irrigated section of a corn field and in 27/40 samples (Ave. 4.4 CFU's/g) from a non-irrigated section of the same field not covered by the pivot. *Aspergillus flavus* was also routinely detected (15 CFU's/g) in overwintered cob residues from the 1990 crop. These cob residues were partially buried in irrigation control plots (8 treatments) to evaluate the impact of irrigation versus drought in producing an *A. flavus* population 'bloom.' We have theorized that summer drought should favor *A. flavus* population build-up in corn fields before the crop has silked.

Repetitive DNA sequences are being used in ecological genetics to distinguish genetically different strains of a fungal species. We have constructed four (4) potential DNA probes for distinguishing biological races (vegetative compatibility groups) of *Aspergillus flavus*. Each probe contains a repeated DNA segment from *A. flavus*, identified in a bacteriophage vector "Lambda Dash" genomic library. These cloned DNA probes will be used to generate DNA fingerprints or hybridization patterns among VCG populations in *A. flavus*.

Artificial baits were successfully applied in field tests to reduce numbers of the dusky sap beetle, *Carpophilus lugubris*, a major insect vector of mycotoxigenic fungi in midwest corn. Long-term release mechanisms for artificial sap beetle attractants were refined in 1991, and new methods for extending the attractancy of natural baits were developed. A potentially new species of nematode that parasitizes the dusky sap beetle was discovered in a pathogen survey. Enzymes from corn kernels involved in browning reactions (which may be associated with resistance to insects and microorganisms) have been partially characterized. Corn volatiles were collected daily from irrigation control plots as part of a larger investigation of the allelochemistry of drought-stressed corn and its attractiveness to corn insects.

Germplasm Sources and Selection for Resistance to Field Aflatoxin Contamination in Maize Breeding Populations

N. W. Widstrom

Insects and other environmental stresses influence both the infection and contamination processes, but our breeding effort has been focused on measurement of resistance to field aflatoxin accumulation. Early measurements clearly indicated the differences in resistance to aflatoxin contamination among commercial hybrids. Laboratory evaluations of whole kernels indicated that at least a portion of this resistance has a chemical basis. Many hybrids have a combination of factors such as stress resistance, insect resistance, tight husks or chemical resistance that contribute to their total resistance. The rate of accumulation of aflatoxin for a hybrid is unique to that hybrid, however, and differences in resistance measured at kernel maturity are a function of that unique rate of accumulation. Additive genetic variation for resistance to aflatoxin contamination exists among maize inbreds. A breeding population was formulated from the best performing inbreds and the best performing commercial hybrids. Two cycles of S_1 selection have been completed during which 10% of a population (200-250 S_1s) were selected for recombination. Heritability estimates varied from 18 to 48%, and progress over two cycles of selection was evaluated over a 3-year period and determined to be a reduction in contamination of approximately 100 ng g^{-1} per cycle.

USDA Aflatoxin Workshop

Atlanta, GA October 21, 1991

Ecology of Aspergillus flavus in Iowa corn fields.

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Most of the information available on the epidemiology of *Aspergillus flavus* in corn has been generated in southern production areas. This may not necessarily be relevant to the northern corn belt. This study involves an ecological study of the fungus in Iowa corn fields as it relates to tillage and inoculum sources.

The first component of the work was a continuation of the biannual monitoring of of 40 fields in Iowa that experienced significant aflatoxin contamination in 1988. Samples of soil and corn debris were taken in the spring of 1991 and tested for the presence and characteristics of *A. flavus*. The pathogen was recovered from 0.04 % of corn crop residues and soil in from almost all of the fields. Approximately half of the isolates produced aflatoxin in culture and 11-20% produced microsclerotia. Results were in close agreement with previous survey data from these fields.

The second component of the study was carried out in permanent conservation tillage plots in northeast Iowa comprising one acre plots under different tillage and rotational practices. These plots were originally established in 1977, and, in 1979 and 1980, *A. flavus* was quantified in the soil, crop residues, new leaf tissue and air of the plots under conventional tillage or no tillage and in continuous corn or soybeans/corn rotational systems. The study was repeated in 1991. Similar results were obtained in all three years of the study. The pathogen was recovered from all four sources at low levels, but there was no consistent evidence to relate populations of the fungus to rotation or cultural practice. Further studies will be needed in the plots to confirm this. The data from the tillage plots and field surveys suggest that there is a low level background population of the fungus in Iowa corn fields.

The third component of the study investigated deposits of waste corn in the proximity of corn cribs and storage bins as potential sources of inoculum. Kernels with sporulating colonies of *A. flavus* were found in piles of corn at the base of six cribs and under the loading port of three bins. As many of 100% of kernels were sporulating at some locations. Airborne spores were trapped in the proximity of the bins or cribs at rates as much as 20 times higher than those recorded in corn fields. Nitidulid beetles were observed crawling on sporulating colonies of *A. flavus* on the waste corn, and the pathogen was isolated from as many 100% of those trapped within a few yards of bins. Considerable variation was found in the degree of infestation of the corn at the locations. It was evident, however, that lower contamination occurred at the sites where weeds were controlled around the cribs or bins. Waste corn clearly is a significant inoculum source from *A, flavus*. Efforts now must be made to determine significance of these inoculum sources with respect to colonization of plants in neighboring corn fields.

Plant Stress and Aflatoxin Contamination of Corn in South Texas

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Over the last 10 years, the Lower Rio Grande Valley of Texas has annually produced more than 100,000 acres of corn. A majority of the corn crop used for human consumption (food corn) is produced in three counties located along the southernmost border of the state. All of the corn in these counties is produced on irrigated land with water obtained from the Rio Grande river. Just across the border in Mexico, the annual production of corn ranges from 250,00 to 350,000 acres. Essentially all of the food corn produced in the Valley is purchased by Azetca Milling. Azteca has located a $25 million facility in the Valley with a capacity to process 200 million pounds of corn. They also purchase an additional 100 million pounds for export. The purchase price for 1991 was between $6.50 and $7.00 per hundred weight for corn meeting minimum standards for aflatoxin contamination. The LRGV has experienced a serious drought during the last 3 years prior to 1991 in which the accumulated average rainfall deficit for May, June, and July varied for 3.5 to 7.6 cm. During the same period, the accumulated deviations of maximum daytime temperatures were 3.3 to 4.7°C above normal. The rainfall improved in 1991 while the accumulated deviations of the maximum temperature remained 2.2°C above normal. The amount of food corn within the limits for aflatoxin decreased from 100% in 1988 to less than 5% of the total crop in 1989. The reduced production of corn within the acceptable limits for aflatoxin contamination have continued through 1991. Less than 5 million pounds of corn have been available for milling during each of the last 3 years. Because of the high rate of rejection, commercial producers have converted to other crops, which reduced the total acreage in the LRGV allocated for food corn production to less than 10,000. The last 4 years of increased contamination with aflatoxin are associated with intervals of low rainfall and higher than normal temperatures. In a cooperative study with Dr. Gary Odvody (TAES, Corpus Christi), we found that supplemental irrigation reduces the visible growth of *Aspergillus flavus* and decreases aflatoxin content by approximately 50% over dryland plots. Earworm pressure in the LRGV is intense and results in high rates of kernel damage which is associated with aflatoxin contamination. A team of scientists (S.Lyda, A.Bockholt, R.Meagher, S.Maas, J.Dunlap) have established plots with several varieties of corn to determine the role of earworm, *A. flavus*, variety, and environmental stress in the development of aflatoxin contamination. Water movement, photosynthetic activity, stomatal resistance, and canopy temperature will be monitored in crop plants as well as a number of meteorological variables to quantify the actual level of stress in inoculated plants grown under irrigated and dryland conditions. We intend to identify physiological processes and management practices that discourage aflatoxin production in corn.

Accumulated Deviations From Monthly Means For May Through July In The Texas LRGV

	YEAR						
	85	86	87	88	89	90	91
Rainfall (cm) (7.3,6.6,5.1)[a]	+19.8	+3.5	+2.2	-7.6	-3.5	-4.5	+2.2
Humidity (Rel. %) (75,75,71)	-4.3	+2.3	-5.8	-29.5	+7.0	-10.7	+33.8
Temperature (°C)							
Min (21.2,22.6 23.9)	+0.8	+2.3	+1.7	-3.2	+4.0	+3.3	+2.2
Max (31.8,33, 34.9)	-0.3	+0.4	-0.9	+3.6	+4.7	+3.3	+2.9
Corn Production Exceeding Aflatoxin Limit[b] (%)	0	0	0	0	99	97	96

[a](75-year means for May, June, and July)
[b]Data supplied by Azteca Milling, Edinburg

BIOCOMPETITION

Update on *Aspergillus flavus* populations and prevention of aflatoxins through intraspecific competition.

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Populations of *Aspergillus flavus* are highly variable. We have shown this variability to be reflected both in the ability of isolates from a single field to produce aflatoxins and in average toxigenicities of populations from different fields. This variability provides a resource for selecting *A. flavus* isolates with many potentially beneficial characteristics. We have selected isolates with both reduced toxigenicity and competitive ability under field conditions and utilized these in our continued efforts to develop techniques to modify *A. flavus* populations in agricultural fields in order to increase the proportion of strains which are atoxigenic and thereby reduce aflatoxin contamination of crops.

In two years of field experiments, in different fields, we have successfully displaced endemic toxigenic strains of *A. flavus* during infection of the developing cotton crop. Displacement was achieved by applying wheat seed colonized by an atoxigenic strain of *A. flavus* to soil beneath the crop canopy prior to first bloom. Displacement was associated with significant reductions in aflatoxin contamination in both years without an increase in boll infection as measured by bright-green-yellow-fluorescence. Furthermore, no increase in the number of *A. flavus* propagules on the mature crop was associated with the displacement. In both years the concentration of toxin in the infected cottonseed was inversely correlated with the incidence of the released atoxigenic strain. Replicate plots with very high incidences of the atoxigenic strains had very low toxin and those with low incidences had high toxin.

In a separate test, spread of a released strain from a single release focus was tested and partial displacement of endemic strains in infected locules was shown to occur up to 100 yards away. In additional multi-year studies, application of atoxigenic strains was shown to cause long term population shifts in the *A. flavus* soil population of an agricultural field and the distribution of three atoxigenic vegetative compatibility groups within several southern states was documented.

Improved formulations of *A. flavus* for application of atoxigenic strains to agricultural fields were also investigated. The production of spores by alginate encapsulated mycelia of an atoxigenic strain of *A. flavus* was investigated and compared with production of spores by *A. flavus* colonized wheat seed and sclerotia of the same strain. Alginate pellets proved to be a superior delivery system. Corn cob grits served as an excellent filler for the pellets whereas other traditional fillers had either less favorable or negative effects. Several adjuvants increased spore release from the pellets and wheat gluten was selected as the most useful. Addition of pesticides to pellets was possible without eliminating pellet ability to release spores. This may permit production of pellets resistant to various fungi, bacteria and insects that may reduce pellet efficacy in the field.

Title of the project

Elimination of aflatoxins in Arizona using beneficial bacteria

Investigators

I.J. Misaghi and P.J. Cotty

Effective control measures have not been developed for aflatoxin contamination of cottonseed which is a chronic problem in Arizona. Therefore, our research objective is to develop environmentally safe, effective, and non-chemical procedures for the management of aflatoxin contamination of cottonseeds. Our approach is to use beneficial bacteria to fight off infection of cottonseeds by the aflatoxin-producing fungus.

We recovered more than 800 isolates of endophytic and non-endophytic bacteria from cottonseeds, bolls, stems and from agricultural soils and screened these bacteria initially for their ability to prevent Aspergillus flavus from colonizing seeds. Endophytic bacteria were also tested for their ability to multiply in wounded boll tissue. However, so far none of the several endophytic isolates that multiply at high rates in wounded cotton tissue have shown antagonistic activity against the fungus. Four non-endophytic bacterial isolates were found that could multiply at rapid rates on immature cotton fiber and could protect cottonseeds from A. flavus invasion in vitro and the developing cotton bolls in the field. Seventy-one percent of cotton bolls, inoculated with a toxigenic isolate of A. flavus in the field, became infected and produced locks that did not fluff out (tight locks). The incidence of boll rot in the field was reduced between 55 and 85 percent in bolls inoculated simultaneously with the fungus and any one of the antagonistic bacterial isolates, compared to that in bolls inoculated with the fungus alone. Similar results were obtained in greenhouse-grown plants and in detached bolls.

All four antagonistic bacteria also prevented germination of sclerotia of A. flavus on agar and in sterile soils. Percentages of germination of sclerotia in the presence and absence of antagonistic bacteria were zero and one-hundred, respectively. The results show that aflatoxin contamination of cottonseeds can potentially be managed biologically not only by reducing the amount of boll infection but also by lowering spore release from sclerotia.

Our goals are to perform additional field trials to test the ability of antagonists to prevent natural infection of cotton bolls and to reduce A. flavus spore load in the soil and on the crop. We will also test additional endophytes for antagonistic activity and will develop procedures for application of antagonists to the field.

October 1991

The Use of Biocompetitive Agents for Preharvest Aflatoxin Control in Peanuts

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We initiated studies using a biocompetitive agent (BA) for preharvest aflatoxin control in 1987 when we introduced a non-aflatoxin-producing strain of _A_. _parasiticus_ into the soil of some of our environmental control plots. The first year's results were not conclusive but showed a degree of promise. However, results from the second year showed the non-aflatoxin-producing agent dominated the soil environment and resulted in tremendous reduction in aflatoxin in the edible peanuts compared to expected levels. The presence of high levels of a chemical marker compound (OMS) in the edible and non-edible peanuts showed that the added biocompetitive agent had replaced the wild strains in the contamination process. The results of the third year's studies were equally promising with the added agent still maintaining a high level of dominance in the soil environment.

Last year's study involved the use of a newly developed mutant of _A_. _parasiticus_ applied to four peanut fields (1/2 acre plots/field) and one corn field (1/2 acre plots). The new mutant was applied as the sclerotial stage at a rate of 8-11 lbs/acre shortly after planting time (approximately 2 weeks). The purpose of including a corn field was to determine the effect of applying the BA with the rotation crop followed with peanuts the next year (1991).

The results of these studies were mixed from no apparent control, to partial and excellent control. The reasons for this were not apparent but could be due to the type of propagule applied (sclerotia), effectiveness of the newly developed mutant, or maybe the new mutant would be more effective the second year after application similar to previous plot studies.

Current and future studies involve development of a BA color mutant, best formulation (i.e. sclerotia, mycelian, etc.), best time for application and effect of crop rotation on BA populations.

Biological Control of Aspergillus flavus and A. parasiticus by Paecilomyces lilacinus and Field Ecology of the A. flavus Group Inoculum in Georgia Soils. D. M. Wilson and M. E. Will, University of Georgia, Coastal Plain Station, Tifton, GA 31793.

Biological Control -

There were two objectives for the biological control portion; to determine if P. lilacinus or other potential biological control fungi effectively inhibited the A. flavus group and if P. lilacinus was functioning via mycoparasitism. Laboratory studies using agar cultures and slides and field studies were conducted. Mycelial cultures of A. flavus and A. parasiticus were not greatly inhibited or parasitized by P. lilacinus or the Trichoderma and Gliocladium species tested. Sclerotia of A. flavus and A. parasiticus were readily colonized in the laboratory by P. lilacinus, Trichoderma and Gliocladium. This colonization by isolates of all fungi resulted in diminished germination. Generally the sclerotia remained viable after a second surface disinfection with the exception of two P. lilacinus isolates (NRRL 13874 and 13876).

The field experiments in Georgia and Illinois, where 'teaballs' containing soil and A. flavus or A. parasiticus sclerotia received P. lilacinus inoculum, chitin and/or cellulose amendments were completed in 1991. The ability of A. flavus group sclerotia to germinate gives an indication of the long-term viability. The sclerotia were buried for six months, covering a growing season in both locations, before they were recovered and assessed for germination and colonization. In Georgia 94-98% and in Illinois 96-98% of the sclerotia retrieved were able to germinate on potato dextrose agar. Twice as many sclerotia from the Illinois location were capable of sporogenic germination on soil than from the Georgia location. Sporogenic germination was low for all A. flavus and A. parasiticus sclerotia in 'teaballs' without treatment or amendments only in Georgia. All treatments, P. lilacinus, chitin and/or cellulose, increased sporogenic germination in Georgia but not in Illinois. However, sporogenic germination is misleading because the sclerotia can also germinate by extending vegetative hyphae. When both sporogenic and hyphal germination were measured on soil, 92-98% of the sclerotia from both locations germinated. Non-germinating sclerotia sometimes were colonized with P. lilacinus and other fungi, with the non-toxic A. parasiticus isolate being colonized most often. Soil populations (propagules per gram, ppg) of the A. flavus group were high in all 'teaballs' (1.5×10^4 ppg in GA and 1.9×10^4 ppg in IL) after the six month exposure indicating that sclerotial germination had taken place, perhaps more than once. The experimental observations on the lack of inhibition or parasitism in vegetative growth and the excellent survival of A. flavus group sclerotia in the field can be used to question if there could be meaningful biological control of the A. flavus group by competing fungi.

Field Ecology -

The field ecology portion of this research had two objectives. First, to determine if there were population changes of A. flavus and A. parasiticus in corn and peanut soils throughout the growing season. Second, to determine the primary inoculum source of the A. flavus group in Georgia soils.

Progress on understanding field ecology has been rapid in 1991. However, 1991 was an unusually wet year and studies on differences between irrigated and dryland fields were hampered by weather. Soils from two 1990 corn fields and two 1990 peanut fields were sampled in 1991 to assess A. flavus group inoculum sources and population changes over the 1991 growing season. Overall, soil from the dryland fields planted with corn and peanut in 1990 tended to have higher A. flavus group population counts and organic matter colonization in 1991 than soil from the irrigated corn and peanut fields. No sclerotia were found in any field soil sample. The inoculum reservoir of the A. flavus group in Georgia soils seems to be colonized organic matter and perhaps conidia. A. flavus group soil populations generally increased in both peanut and corn soils over time in 1991.

Nine different culture media were tested to find if A. flavus could be presumptively separated from A. parasiticus in soil samples. Dextrose peptone yeast agar was found to be useful for presumptive identification of A. flavus and A. parasiticus. Studies on 1991 peanut soils are in progress.

Pure and mixed cultures of A. flavus and A. parasiticus were grown in liquid culture to assess aflatoxin B_1, B_2, G_1 and G_2 production. Aflatoxin G_1 and G_2 production by A. parasiticus was diminished by the presence of only 12.5-25% A. flavus in the inoculum. This effect could be due to one or more factors. A. flavus may have grown and synthesized aflatoxins more rapidly than A. parasiticus, A. flavus may have produced substances inhibitory to the production of G_1 and G_2, or A. flavus may have the ability to metabolize the G aflatoxins.

Evaluation of Microorganisms from Pistachio Orchards for Antagonism to *Aspergillus flavus/parasiticus*.

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In 1990, potential antagonists against *Aspergillus flavus* were isolated from pistachio orchards in California using an Andersen Sampler. Out of 95 potential antagonists that were selected, most produced large inhibition zones against *A. flavus* in potato-dextrose agar. One of these promising antagonists was selected for two further tests using pistachio fruits. This bacterial antagonist resulted in less growth of *A. flavus* in one of two tests and some reduction in aflatoxin production in both tests using pistachio fruits.

In 1991, we decided that more antagonists were needed representing a broader range of organisms. Approximately 135 new isolates, including both bacteria and several genera of fungi, were isolated from several sources in pistachio orchards. These potential antagonists were tested for inhibition of *A. flavus* in various media. Many antagonists performed well in inhibiting *A. flavus*, although the inhibition was less in some media. The most promising of these antagonists were tested using pistachio fruits. Seven different antagonists (four genera of fungi and three isolates of bacteria) performed well in inhibiting growth of *A. flavus* in all tests. A field test is now in progress.

Resistance and Biological Control of Aflatoxigenic Fungi in the Almond, Pecan, Pistachio and Walnut

Principal Investigators: N.F. Sommer, UC-Davis and T.J. Michailides UC-Berkeley (Parlier)

Co-Investigators: Gale McGranahan, Dan Parfitt and Tom Gradziel, Nut crops improvement, UC-Davis

Cooperators: R.J. Fortlage and H. Ahmadi.

Studies have concentrated on the following: A) Means of exclusion of *Aspergillus flavus* or *A. parasiticus* from nuts, and B) Use of biocontrol agents against the aflatoxigenic fungi.

Exclusion. In studies underway, it appears that nuts are protected by three or possibly four protective structures: the hull, shell and seed coat. Current tests with almond, pistachio and walnut suggests that the kernel tissue immediately below the seed coat is also highly resistant to aflatoxigenic fungi.

Effectiveness of kernel protecting structures may vary widely between cultivars. In almonds, for example, some cultivars, such as 'Mission' have a hard, thick hull and aflatoxin is rare. The 'Nonpariel', on the other hand, has a thin shell that does not completely enclose the kernel. Aflatoxin is a problem with that. Unfortunately, 'Nonpariel' is of high quality and the most popular cultivar representing about half of the California production. It seems likely, however, that a high quality nut could be developed with a better shell and other protective layers. Such protection is known to reduce entrance of peach twig borers and Navel Orange Worm as well as aflatoxigenic fungi.

Pistachio nuts are protected from aflatoxigenic infections or by a hull that normally protects the kernel until it is ripe. A defect, "early split", causes some kernels to be susceptible to insect infestation and/or infections by aflatoxigenic fungi. The reduction of "early splitting" would seemingly result in important reductions in aflatoxin.

Studies are underway on means of entrance of aflatoxigenic fungi into walnuts and pecans.

Biocontrol. Studies have centered on yeasts both from orchards and from culture collections. Among several hundred tested, best results thus far have been given by *Rhodosporidium diobovatum* Newell & Hunter [Anamorph *Rhodotorula glutinis* (Fres.) Harr.] and *Cystofilobasidium capitatum* (Fell et. al) Ober. et. al. [Anamorph *Cryptococcus infirmo-miniatus* (Ok.) Phaff & Fell.] In tests with almonds we achieved 80-90% reduction of *A. flavus* infections. That level of suppression does not appear encouraging to us.

DEVELOPMENT OF *IN VIVO* ASSAYS FOR BIOLOGICAL CONTROL AGENTS AGAINST AFLATOXIGENIC FUNGI ON PEANUT

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ABSTRACT

Two *in vivo* assays were developed for screening candidate biological control agents of aflatoxigenic fungi. The assays consisted of peanut seeds and root radicles inoculated with *Aspergillus flavus*-type fungi. An inoculum concentration of log 4.3 conidia per seed or radicle was selected based on time required for first appearance of mycelia and for complete coverage of the seed or radicle. Screening of bacteria with known biological control activity in a cotton seedling damping-off system revealed that several bacterial strains prevented peanut seed and radicle colonization by *A. flavus*. A collection of bacteria from peanut geocarpospheres is now being screened in the assays. Preliminary determinations of bacterial effects on aflatoxin production revealed that some bacteria reduce the total aflatoxin levels and cause shifts in the relative levels of aflatoxins G1, B1, G2, and B2.

Poster

Antagonistic effect of chitinolytic bacteria on _Aspergillus_.

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Eight chitinolytic bacteria were tested for antagonistic effects against _Aspergillus flavus_ and _A. parasiticus_. Bacteria were grown on solid media in the presence and absence of chitin for 3 days prior to the introduction of fungi. Two of the 8 bacteria, _Serratia marcescens_ and _Aeromonas hydrophila_ inhibited growth (in the region of chitinolytic clearing surrounding the bacterial colony) of both _Aspergillus_ sp. in the presence of chitin, but the latter bacterial species also inhibited fungal growth in the absence of chitin. All other chitinolytic bacteria did not affect fungal growth although chitinolytic clearing zones were present. This may indicate chitinases produced by the different bacteria have different enzymatic properties and antifungal activities against _Aspergillus_ sp. In addition, chitinolytic activity of _A. hydrophila_ appears to be expressed constitutively. Studies are under way to test activities of plant chitinases expressed in _E. coli_ against _Aspergillus_ sp. The ultimate goal of the project is to express genes encoding chitinases and other antifungal proteins in seed (or other plant tissues) to prevent invasion of host plant tissues by aflatoxin producing fungi.

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Hydrolytic Enzymes Expressed by *Paecilomyces lilacinus* During Growth on Sclerotia of *Aspergillus flavus*.

Sclerotia of *Aspergillus flavus* may be important in the annual persistence of the fungus in the soil. Natural turnover of *A. flavus* sclerotia in soil has been found to be associated with parasitism by *Paecilomyces lilacinus*. As a preliminary investigation of the role of degradative enzymes in mycoparasitism of *A. flavus*, we quantitated a variety of enzymes from *P. lilacinus* cultured in vitro on sclerotia of *A. flavus*. All strains secreted low levels of cellulase, chitinase, and β-(1,3)-glucanase. Some, but not all, strains also secreted β-(1,4)-glucanase and α-(1,6)-glucanase ("dextranase"). All strains also made N-acetyl glucosaminidase ("chitobiase" or "exochitinase"). Much higher levels of proteases were found, specifically chymoelastase and chymotrypsin activities. Trypsin appears not to be an important product. These results offer initial direction for studies to identify determinative virulence and host range factors in mycoparasitism of *A. flavus*.

TOXIN BIOSYNTHESIS

Control of Aflatoxin Contamination: Characterization of the Toxin Biosynthetic Grid and Governing Genes

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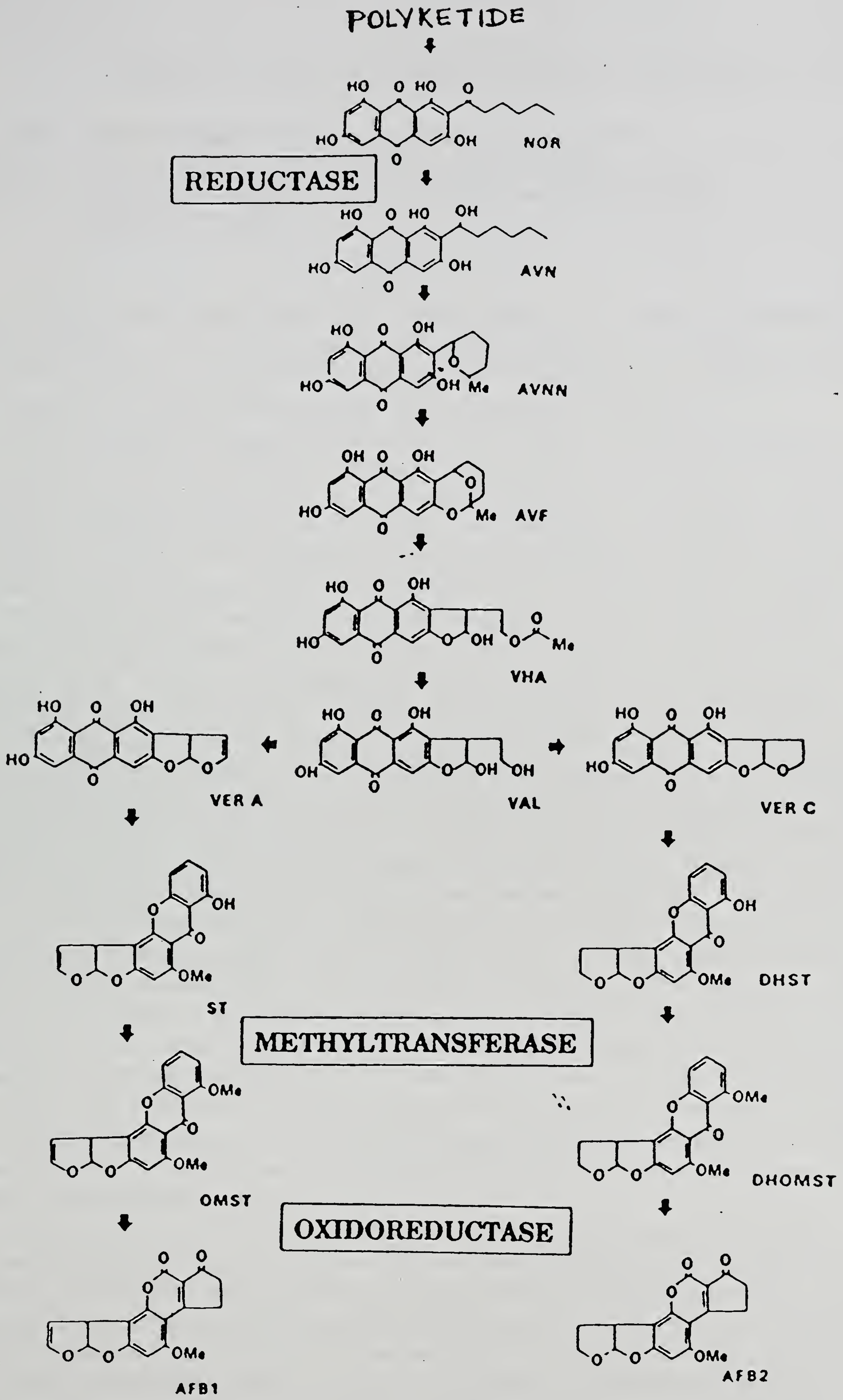
Determination of the mode of toxin formation depends entirely on the understanding of various chemical and biochemical steps in the synthesis of aflatoxins. Such knowledge requires identification of intermediates compounds and enzymes required for the toxin biosynthesis. Once this pathway is clearly understood, a chemical or genetic means of preventing toxin formation could be developed for all the crops, i.e. corn, cotton, peanuts and treenuts. Perhaps the one feature all aflatoxin- contaminated crops have in common is that the regulatory controls that govern the complex toxin biosynthetic process will be very similar.

In imperfect fungi (e. g. A. parasiticus), genetic investigations require production of mutants with auxotrophic or morphological markers. Several aflatoxin blocked mutants of A. parasiticus have been isolated and characterized in this laboratory. Using these mutants a number of important discoveries have been made: (1) several intermediates in the biosynthetic pathway have been identified (Fig.); (2) presence of several specific enzyme systems in cell-free fungal extracts have been detected (Fig.); (3) induction of aflatoxin biosynthetic enzymes in fungal fermentation was exclusively linked to the onset of secondary metabolism in intact fungal cells; (4) several enzymes involved specifically in the pathway have been purified to homogeneity and characterized; (5) oligonucleotide probes have been developed based on the primary amino acid sequence of these proteins; antibody probes have also been developed; (6) these discoveries have resulted in the identification of two genes (for methyltransferase and reductase) involved in aflatoxin biosynthesis by probing the cDNA and genomic libraries prepared against A. parasiticus DNA; (7) the molecular regulation of one of the genes (for reductase) has been determined; and (8) methodology has been developed to determine the location of these genes on A. flavus and A. parasiticus chromosomes.

The specific areas of research that appear to offer the greatest opportunity include: (1) characterization of conditions or compounds that induce gene expression for the enzymes; (2) adaptation of the DNA probes to identify complementary areas of fungal DNA and their association with toxin synthesis; (3) acquisition of pathway information by pertinent antibody and DNA probes in mutants, non-producing and toxin-producing isolates of A. flavus and A. parasiticus; (4) development of stable, non-producing fungal isolates that could be introduced into agroecosystems without the hazard of subsequent incorporation of genetic information for toxin production; (5) identification of constituents of developing crop seed that function as bioregulatory agents in fungi and genetic expression of enzymes involved in aflatoxin biosynthesis.



Scheme of chemical intermediates and enzymes involved in aflatoxin biosynthesis: NOR, Norsolorinic acid; AVN, averantin; AVNN, averufanin; AVF, averufin; VHA, versicolorin hemiacetal acetate; VAL, versiconal; Ver A, versicolorin A; Ver C, Versicolorin C; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin.

REPORT TO THE AFLATOXIN TECHNICAL ADVISORY GROUP

Project Title:	Characterization of Genes for Aflatoxin Biosynthesis
Investigator:	Gary A. Payne, North Carolina State University
Cooperators:	Drs. Deepak Bhatnagar and T. E. Cleveland, USDA/SRRC
Date:	October 25, 1991

The goal of this research project is to isolate genes in the aflatoxin biosynthetic pathway and study their regulation. We hope to use the information gained in these studies to devise strategies that either block the pathway at a specific step or disrupt its regulation. The long term goal is to engineer plants containing a compound that prevents the accumulation of aflatoxin.

We have isolated two genes that appear to be involved in aflatoxin formation. One of these genes, designated CW3, was isolated by complementary DNA hybridization. This gene is expressed only during aflatoxin formation and maximum expression of this gene is correlated with maximum accumulation of aflatoxin. CW3 appears to be a dehydrogenase as its deduced amino acid sequence is 83% similar to an alcohol dehydrogenase from *Aspergillus nidulans*, and CW3 has the conserved binding sites present in dehydrogenases for zinc and NADH. The role of this gene in aflatoxin biosynthesis is not known. It may be directly involved in aflatoxin biosynthesis as there are several biosynthetic reactions in the pathway catalyzed by dehydrogenases. Alternatively, the gene may not code for a pathway enzyme, but it may code for an enzyme in another pathway that is required for aflatoxin biosynthesis. We have several strategies planned to determine the role of this gene in aflatoxin biosynthesis.

The second gene we have isolated appears to be directly involved in aflatoxin biosynthesis. The gene was identified by its ability to restore aflatoxin-producing ability to a non-aflatoxin producing strain. We have designated this gene *afl-2*, because it complements the mutated *afl-2* gene in *Aspergillus flavus* strain 650-33. We do not know the product of the gene, but we have good evidence for the location of the gene in the pathway. We moved, by parasexual recombination, a defective *afl-2* gene into another strain of *A. flavus* that accumulates the pathway intermediate norsolorinic acid (NOR). The resulting double mutant did not produce NOR. Transformation of the double mutant with the *afl-2* gene restored NOR production, indicating that the block is before NOR. As NOR is the first stable intermediate that accumulates in the pathway, *afl-2* must be involved in early steps of the pathway. Additional evidence for an early site of *afl-2* in the pathway comes from metabolite feeding studies done by Drs. Bhatnagar and Cleveland. Most strains blocked in aflatoxin biosynthesis possess most of the pathway enzymes and, if fed pathway intermediates after the block, these strains can convert the intermediates to aflatoxin. Strain 650-33, with a defective *afl-2* gene, did not convert any of six pathway intermediates to aflatoxin. Transformation of 650-33 with a functional *afl-2* gene restored enzyme activity and aflatoxin formation. Thus, not only is the gene early in the pathway, but a block at the *afl-2* site appears to prevent the expression of other pathway genes.

Our current studies are directed at examining the promoter region of the *afl-2* gene. Because this gene is early in the pathway, it is a prime target for studies directed at aflatoxin disruption. These studies are aimed at our long-term goal of finding a compound that can be engineered into a plant to disrupt aflatoxin formation. We also have immediate plans to use the *afl-2* gene. Once the promoter region of the gene is defined, we plan to fuse the promoter with the reporter gene GUS and develop a rapid and sensitive assay for evaluating plant genotypes for resistance to aflatoxin accumulation. The product of the GUS gene catalyzes the cleavage of 4-methyl umbelliferyl β-D-glucuronide to produce a product that can be quantified by fluorescence. This construct will allow us to identify plant metabolites that either induce or prevent the induction of a gene for aflatoxin biosynthesis. Because this assay directly measures the expression of a gene and not the final product of a complicated pathway, it will be more sensitive than current techniques and it will not be complicated by the effects of the test compounds on primary metabolism.

PRODUCTION AND CHARACTERIZATION OF ANTIBODIES AGAINST NORSOLORINIC ACID REDUCTASE

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ABSTRACT

Norsolorinic acid reductase (NSR) is one of the key enzymes in the early stage of aflatoxin biosynthetic pathway. To further our understanding of the structure and function of the enzyme, attempts to produce both monoclonal and polyclonal antibodies were made. An indirect ELISA was established for measurement of the antibody titer using partially purified NSR as coating antigen and a second antibody-peroxidase conjugate as indicator. Polyclonal antibodies against NSR were demonstrated in rabbits 10 weeks after the animals were immunized with a purified enzyme preparation (2 bands). Western blot analysis revealed that the antibodies reacted with two major proteins of 48 and 38 kdaltons. The partially purified antisera (ammonium sulfate precipitate) were able to inhibit the enzyme activity associated with both proteins. Further separation of the 48 kd and 38 kd proteins was achieved by Sephadex G-100 gel filtration. The 38 kd protein was then conjugated to Sepharose gel, the gel was then used as an affinity column for the separation of antibodies specific for the 48 and 38 kd proteins. Further analysis revealed that antibodies against the 48 kd protein inhibited enzyme activity. The enzyme preparation containing primarily the 48 kd protein was selected to immunize BABL/c mice for production of monoclonal antibodies. A total of 12 hybridoma cell lines producing monoclonal antibodies against various proteins were obtained. Detailed properties of the monoclonal antibodies and their binding with the enzymes were examined by analysis of affinity chromatography, Western blots, enzyme inhibition and other immunochemical studies.

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Abstract: Characterization of 3 genes associated with aflatoxin biosynthesis in _Aspergillus_ _parasiticus_

A. The _Ver_-1 gene: associated with conversion of versicolorin A to sterigmatocystin (two intermediates in aflatoxin B1 biosynthesis)

The gene encoding OMP-decarboxylase (_pyr_G) was mutated in a strain of the filamentous fungus _Aspergillus_ _parasiticus_ ATTC 36537 which is unable to convert the aflatoxin intermediate versicolorin A to sterigmatocystin. We transformed this aflatoxin blocked mutant strain with DNA from the "wild type" toxin producer _A_. _parasiticus_ NRRL 5862 (SU-1) contained in a cosmid library using the homologous _pyr_G gene as a selectable marker. Prototrophic transformants were selected directly on coconut agar medium (CAM) and were initially screened for aflatoxin production by U.V. fluorescence on the CAM. One transformant clone produced a fluorescent blue pigment later confirmed by thin-layer chromatography and enzyme linked immunosorbent assay to contain aflatoxin B1 and G1. A DNA fragment linked to the ampicillin resistance gene from the integrated cosmid was recovered from this transformant by marker rescue. This DNA fragment was able to restore aflatoxin production in _A_. _parasiticus_ ATTC 36537 to levels comparable to "wild type" in thirty percent of the total transformants. The complementing activity of the _ver_-1 gene has been localized to a 1.6 Kb DNA restriction fragment. Northern analysis revealed that this fragment encodes a 1.0 Kb transcript.

B. The _nor_-1 Gene: associated with conversion of norsolorinic acid to averantin in aflatoxin B1 biosynthesis

A second cosmid library was constructed by inserting genomic DNA isolated from the wild-type aflatoxin-producing strain of _Aspergillus_ _parasiticus_ NRRL 5862 (SU-1) into a cosmid vector containing a homologous nitrate reductase (_nia_D) gene as a selectable marker. One cosmid was isolated which complemented an aflatoxin-deficient, nitrate-nonutilizing mutant strain, _A_. _parasiticus_ B62 (_nor_-1, _nia_D), to aflatoxin production. Deletion and complementation analyses showed that a 1.7 kb _Bgl_II-_Sph_I DNA fragment isolated from this cosmid was responsible for renewed aflatoxin production. Northern hybridization analyses revealed that the major RNA transcribed from this DNA fragment containing the _nor_-1 gene was 1.4 kilonucleotides in size.

C. The PKS gene (polyketide synthetase)

We have recently cloned another DNA fragment from _Aspergillus_ _parasiticus_ which is also likely to encode a key enzyme in the aflatoxin biosynthetic pathway. We used a DNA probe from a distant relative of _Aspergillus_ to screen the _A_. _parasiticus_ genomic DNA library. The DNA probe was a small fragment (1.2 Kb) of a cDNA clone which encodes the polyfunctional polyketide synthetase (PKS) enzyme from _Penicillium_ _patalum_ (kindly provided by E. Sweizer). _P_. _patulum_ synthesizes a tetraketide (4 acetic acids) called 6 methyl salicylic acid which is the direct precurser to patulin, a potent mycotoxin. Aflatoxin also is a polyketide (decaketide) derived secondary metabolite. People have speculated for several years on the presence of a polyketide synthetase enzyme in _Aspergillus_. We suspected there might be enough similarity between the _P_. _patulum_ PKS gene and the putative _A._

parasiticus PKS gene that we could successfully isolate the A. parasiticus gene by hybridization to the heterologous gene probe. We have isolated a 1.9 Kb DNA restriction fragment (Hind III) from our A. parasiticus genomic DNA library which hybridized strongly at relatively high stringency to the P. patulum probe. A 680 bp DNA fragment was subcloned from the 1.9 Kb Hind III fragment and the nucleotide sequence determined. The predicted amino acid sequence of the putative A. parasiticus PKS was compared with the published amino acid sequence from Penicillium patulum PKS and found to be 75% similar. Northern analysis of A. parasiticus RNA using the 680 bp PKS fragment detected a transcript of 10 Kb. This data is preliminary and must be confirmed. However the size of this PKS transcript is not unreasonable because we anticipate that this gene encodes a large polyfunctional protein analagous to PKS in P. patulum (~6 Kb transcript). If this is the A. parasiticus PKS gene as we suspect, it may be quite significant because this polyfunctional protein is responsible for carrying out the first biosynthetic steps in the aflatoxin pathway. In general, regulation of biochemical pathways occurs at the first enzymatic step. We therefore may have a tool in hand to understand one key regulatory step (among many regulatory steps).

We have shown recently that a β-tubulin gene which was cloned from a benomyl resistant mutant strain of A. parasiticus can confer resistance to benomyl (a fungicide) in a sensitive A. parasiticus strain (CS10) after being introduced into protoplasts by transformation. This is important because it may allow us to use this "dominant selectable marker" to directly transform wild type strains of A. parasiticus (which are benomyl sensitive) with gene disruption constructs to induce directed mutations in aflatoxin biosynthetic genes. We can then study phenotypic changes in growth and development which may occur when strains which normally synthesize aflatoxin B1 are no longer able to because of a gene disruption in a single gene involved in aflatoxin biosynthesis.

We also demonstrated recently that we are able to disrupt the function of a model gene, niaD, which encodes nitrate reductase. This is significant because gene disruption is a key protocol to introducing directed mutations in aflatoxin biosynthetic genes in a wild type genetic background (as described above) to study potential function of these secondary metabolites in cell growth and development. This technology also will enable us to genetically engineer stable nontoxigenic biocontrol strains of A. parasiticus which can be tested eventually in field trials for their efficacy (through biological exclusion) in preventing toxigenic strains from infecting crops and producing aflatoxins.

INCREASING HOST RESISTANCE TO AFLATOXIN PRODUCING FUNGI

Development of a Gene Transfer System for Peanut

A. K. Weissinger, J. A. Schnall,
P. Ozias-Akins and T. E. Cleveland

We are working to develop a transformation system for peanut (Arachis hypogaea L.) via microprojectile bombardment of plasmid DNA into intact tissues. Potential target tissues were identified by bombarding a number of different intact organs and cultured tissues with microprojectiles carrying plasmid DNA encoding both beta- glucuronidase (GUS) and neomycin phosphotransferase (NPT II) chimeric genes. Transient GUS expression in leaflet and shoot-apex of mature zygotic embryos, and in cultured somatic embryos demonstrated that DNA could be introduced, and that genes driven by the CAMV 35S promoter are expressed in these tissues.

The leaflet explant provides ideal geometry for bombardment and selection, and is capable of plant regeneration. Leaflets were found to be susceptible to kanamycin. Bombarded leaflets were placed on non-lethal levels of kanamycin (50mg/L) 1 to 4 days after bombardment. Slow-growing calli developed on most explants. After protracted selection, small, rapidly-growing, chlorophyllous cell masses developed on some explants. These continued rapid proliferation on 200mg/L kanamycin. All callus lines tested contain integrated DNA sequences of both NPT II and GUS. All transformed calli produced NPT enzyme at various levels, and half also produced GUS enzyme. Culture conditions have been optimized so that it is now possible to recover fertile plants from leaflets of all botanical types of peanut. While stably transformed tissues have been recovered, no stably transformed plants have yet been identified. Large-scale bombardment experiments are now underway in an attempt to recover transformed plants which may be produced only rarely in this system. Although transgenic plants have not been recovered, this system can now be used routinely for the production of transformed peanut callus. This provides us with a test system in which alien genes related to defense against Aspergillus spp. can be expressed. Such transgenic tissues can then be used to test for efficacy of the gene product against the target fungus.

We are now engaged in optimization of bombardment, selection and regeneration protocols for somatic embryos. Because these explants are capable of more frequent regeneration, optimization of this explant system appears to offer the highest probability of producing transgenic plants.

IN VITRO PROLIFERATION AND REGENERATION OF PEANUT TISSUES SUITABLE FOR TRANSFORMATION

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Arthur Weissinger, North Carolina State University, Raleigh
21 October, 1991

A collaboration was established approximately a year ago between the group in Tifton whose emphasis is on regeneration from tissue cultures of peanut, and the group in Raleigh whose expertise is in the use of biolistic particle delivery for transformation of plant tissues. Our work over the past few months has concentrated on optimization of conditions for particle bombardment of embryogenic callus cultures and for selectable marker strategies.

Embryogenic callus cultures can be initiated from immature cotyledons and shoot tips on media containing the growth regulator picloram. We have compared the potential for somatic embryo initiation, embryogenic callus formation, and plant regeneration among seven peanut genotypes, three spanish ('Toalson', 'Dixie Spanish', 'Spancross'), one valencia ('Georgia Red'), and three virginia/runner ('Tifrun', 'Sunrunner', 'Virginia Runner G-26'). Significant differences were observed among genotypes for all traits examined; however, all were capable of somatic embryo formation and plant regeneration to some degree. Long-term (18-month-old) cultures of 'Toalson' are still capable of regenerating plants.

Most of the bombardment experiments conducted to date have involved 'Toalson' because of the availability of a large number of embryogenic cultures. In order to determine if developmental stage can affect transient expression, four classes of somatic embryo/callus were separated and bombarded. The classes were a) torpedo-shaped somatic embryos, b) somatic embryos with differentiated apices, c) embryogenic callus, d) non-embryogenic callus derived from embryogenic cultures. Transient expression, as measured by number of ß-glucuronidase (GUS)-positive foci, was highest in differentiated-apex embryos and embryogenic callus. Very low expression was observed in non-embryogenic callus. Preliminary experiments indicate that GUS expression can persist over an extended period of time relative to the normal peak of 24-48 h post-bombardment typically observed in other tissue culture systems.

Three large-scale bombardment experiments, two with a hygromycin-resistance gene and one with a kanamycin-resistance gene, have been conducted. Tissues are currently under selection on antibiotic-containing media. Under selective conditions, most of the tissues senesce, rapidly on hygromycin-containing medium and slowly on kanamycin-containing medium. A few surviving calli currently are being increased in order to obtain sufficient material for determination of stable transformation by the polymerase chain reaction and southern analysis.

Candidate genes for use in genetic engineering of resistant crops.

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Technology is being developed in this laboratory and in the laboratories of our cooperators for incorporation of genes into plants for resistance against aflatoxin producing fungi. Development of this technology depends on the accomplishment of the following goals: 1) the identification of gene products that inhibit growth or aflatoxin production by aflatoxigenic fungi; 2) cloning of the genes for these inhibitory compounds; 3) development of techniques to stably transform plants with inhibitor genes; and 4) identification of gene regulatory domains to be used in the construction of chimeric genes with site/tissue specific expression.

Potential fungal growth inhibitors that are being tested in this laboratory and in cooperators' laboratories are enzymes that hydrolyze fungal cell walls of _A. flavus_ such as chitinases and glucanases, peptides that lyse cell membranes, and inhibitors of fungal cell-wall degrading enzymes (pectinases and proteases). Certain bacterial chitinases appear to have antifungal activity against _A. flavus_ and _A. parasiticus_ (see poster abstract, next page). Lytic peptides (e.g. cecropins) also have been demonstrated to have antifungal activities. A polygalacturonase inhibitor isolated from orange mesocarp was shown to inhibit _A. flavus_ pectinases. Pectinases are cell wall degrading enzymes that were shown in our laboratory to facilitate invasion of cotton boll tissues by this fungus. Proteinase inhibitor II (from potato) was shown to inhibit proteolysis of gelatin (a high hydroxyproline containing protein similar to proteins in plant cell walls) by _A. flavus_ protease. Experiments are underway to test pectinase and protease inhibitors for their effects on fungal growth.

Genes for chitinases (S. Tuzun, Cooperator, Auburn University) and protease inhibitors (this laboratory) have been cloned and are being used to design chimeric fungal inhibitor genes for transformation of plants; pectinase inhibitor genes will be cloned if these inhibitors are shown to inhibit fungal growth and used in genetic engineering strategies. A wound-inducible promoter (isolated from the proteinase inhibitor II gene) and a cottonseed storage protein promoter are being used to construct chimeric genes which will yield site/tissue specific expression of antifungal enzymes (such as chitinases) in transformed plants; chimeric genes now available for testing in our laboratory include certain fungal inhibitor genes linked to the cauliflower mosaic virus promoter (yielding constitutive expression of genes) and to the wound-inducible promoter. Genes engineered to yield maximum antifungal activity and site/tissue specific expression will be used in ongoing experiments to transform cotton and peanut (in cooperation with C. Chlan, University of Southwestern LA and A. Weissinger, North Carolina State University, respectively) and to enhance resistance in these crops to invasion by _A. flavus_ and _A. parasiticus_.

BREEDING FOR RESISTANCE

Breeding Peanut for Resistance to Preharvest Aflatoxin Contamination. C. C. Holbrook[1], D. M. Wilson[2], M. E. Matheron[3], W. F. Anderson[1] and M. E. Will[2]. [1] USDA-ARS, Tifton, GA; [2] Univ. of Georgia, Tifton, GA; [3] Univ. of Arizona, Somerton, AZ.

Preharvest aflatoxin contamination (PAC) is one of the most significant challenges facing the U. S. peanut industry. The development of peanut cultivars with resistance to PAC would be a valuable tool in reducing the problem. There are two requirements for developing PAC resistant cultivars. First, there must be genes for resistance to PAC. Second, there must be a reliable and efficient screening technique which can be used to identify material which contains these genes. The objectives for 1990 were developed to address these requirements. The first objective was to select a core collection for peanut which can be used to efficiently identify genes for resistance in peanut. Multivariate statistical analysis was used to select a core collection of 831 accessions from the U. S. germplasm collection (7,432 accessions) of peanut. Examination of data for six phenotypic traits indicated that the core collection is representative of the entire collection and that the genetic variance which exist for each trait in the entire collection has been maintain in the core collection. The use of this core collection should greatly speed the process of identifying genes for resistance to PAC. The second objective for 1990 was to begin research on development of a reliable and efficient screening technique. Ten methods of inoculation were examined to determine which one would be best for insuring _Aspergillus_ colonization. The use of corn as an organic carrier for fungal inoculation at the midbloom stage of peanut development resulted in greater and more stable soil population counts of _Aspergillus_. This was reflected in higher colonization of pods and seeds. However, aflatoxin contamination was high in seed from all treatments, probably due to an adequate background population of _Aspergillus_. Three locations were also studied to determine which would provide the appropriate environmental conditions for PAC. Aflatoxin contamination was consistently high in rain protected field plots at Tifton, Georgia in 1990. Aflatoxin was high, but inconsistent in field plots at Yuma, Arizona. Aflatoxin levels up to 2,000 ppb were observed in peanut from Yuma. Aflatoxin was more prevalent in peanut subjected to summer drought than in peanut subjected to a fall stress period. The use of shade cloth to minimize the extreme summer temperature at this location did not result in greater aflatoxin contamination. These results demonstrate that soil temperatures at Yuma do not consistently exceed the range necessary for PAC. The inconsistency in PAC at Yuma may be due to the difficulty in imposing an extended drought on the pods without killing the plants. Aflatoxin was low and inconsistent from greenhouse grown peanuts at Tifton. Studies being conducted in 1991 are aimed at refining the screening techniques for field screening at Tifton and Yuma and for greenhouse screening at Tifton. In addition, approximately one-half of the accession from the core collection are being evaluated for resistance to PAC.

10-22-91

Patterns of aflatoxin contamination among commercial corn hybrids inoculated with _Aspergillus flavus_. 1

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A procedure for identifying _A. flavus_-aflatoxin resistant/susceptible corn genotypes, among 25 inbreds and 35 hybrids, was evaluated in 1990-91 field tests conducted at Bloomington, IL & Union City, TN. Ears in the late-milk to early-dough stage of kernel maturity were wound-inoculated by inserting a pipe cleaner contaminated with _A. flavus_ spores into each of three holes (3-4 mm) punched through the husk with a drill bit that also damaged the underlying kernel(s). At harvest we inspected each wound site for _A. flavus_ sporulation and removed the pipe cleaner and any kernels damaged by the drill bit. Approximately twenty non-damaged kernels surrounding each wound site were removed from the ear and pooled with similar sets of kernels from ears in each test row. The pooled kernel sample was examined for numbers of BGYF kernels and aflatoxin (ppb). At each location we examined four replicate rows of the 60 corn genotypes.

In 1990, hybrids planted in Bloomington showed average aflatoxin values (4 replicate rows) ranging from 26 ppb to 590 ppb, while the same hybrids planted in Union City averaged 130 ppb to 1870 ppb. Inbreds were particularly susceptible to aflatoxin contamination when grown in Union City (range = 670-4600 ppb) with 10 genotypes showing aflatoxin values exceeding 1800 ppb. Patterns of aflatoxin resistance/susceptibility among hybrids were contrasted with each hybrid's reactions to three ear-rot pathogens (i.e. _Gibberella zeae_, _Fusarium moniliforme_, _Diplodia maydis_). Three hybrids rated most resistant to _D. maydis_ and five hybrids rated most susceptible to _F. moniliforme_ had comparatively low aflatoxin values in tests performed in Bloomington, IL. These data with results from 1991 field tests will serve as a basis for genetic improvement of hybrid resistance to _A. flavus_ while providing relevant experimental material for basic research on the basic mechanisms of kernel resistance.

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1. Cooperative research with Dr. Loral Castor, CIBA-GEIGY Seed Division, Bloomington, IL.

Identification of Molecular Markers Associated With Genes for Preharvest Resistance in Corn to Aspergillus flavus and Aflatoxin Production

Progress Report

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The University of Illinois has an extensive collection of inbreds that are being screened for resistance to *Aspergillus flavus* and aflatoxin. These inbreds are being screened as F1 crosses to B73 and/or Mo17. In 1991 1,189 F1 crosses with Mo17 and 978 crosses with B73 were evaluated in two replicates for resistance to *A. flavus*. Twelve to 18 plants in each replicate were inoculated 20-24 days following pollination with an inoculator developed at the University of Illinois. The inoculator is a pinboard with seven rows of 25 pins. Pins are spaced .4 cm apart. In the center of the pins is a larger needle through which 5 ml of a spore suspension containing 1×10^6 conidia per ml is injected under the husk. Thirty to 40 days following inoculation husk leaves were pulled back and ears evaluated for the amount of rot caused by *Aspergillus flavus*. Seventeen F1 crosses with Mo17 and 18 F1 crosses with B73 were selected for further study. Four of the F1 crosses had inbreds that provided resistance to both B73 and Mo17. The remainder of the inbreds provided a high level of resistance only to B73 or Mo17.

The selected F1 crosses have been sent to Hawaii to produce F2 and backcross to susceptible generations. For seven inbreds we hope to be able to generate both F2 and F3 generations. These generations will be evaluated in Illinois in the summer of 1992. In addition, grain from ears of the best F1's are being analyzed for the presence of aflatoxin. Those that have lowest level of toxin and the best resistance particularly to both parents will be selected for additional studies.

SELECTION FOR HEAT-STRESS RESISTANCE AS AN APPROACH TO OBTAIN *ASPERGILLUS FLAVUS* RESISTANCE IN CORN

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Patterns of aflatoxin outbreaks in field-grown maize, as well as experimental results in controlled environment, have lead to the hypothesis that high-temperature stress greatly enhances susceptibility of maize kernels to infection by the toxin producing fungus *Aspergillus flavus*. Selection for heat tolerance in maize could possibly result in lines more resistant to *A. flavus* infection. Germplasm of open-pollinated maize lines traditionally grown by Native Americans in the desert southwest have undergone centuries of indirect selection for high-temperature performance. In 1988 Dr. Pratt began crossing these lines with high-yielding Corn Belt lines and has demonstrated segregation for heat stress in 1991 field trials. Kernels from desert southwest and Corn Belt lines will be evaluated for their tolerance to 'stressful elevated temperatures' (34 C day/ 30 C night) using *in vitro* kernel culture. *In vitro* culture will allow evaluation of kernel development free from the confounding effects of drought stress, commonly associated with heat stress. Kernels grown in culture are equivalent in size and appearance to kernels harvested from plants grown in the field. Therefore, initial selection for heat-tolerance among F_2 segregants can be performed in culture, exclusive of field trials. Kernels will be cultured for six days at non-stressful temperatures (30 C day/ 20 C night) to establish kernel growth, followed by continuous incubation under conditions of elevated temperature stress. Some kernels will be harvested at 20 days post-pollination (DPP) in order to evaluate the metabolic effects of heat stress on heat-tolerant and heat-resistant lines and to make preliminary evaluations of the physiological mechanism of heat-stress tolerance. Analyses of immature kernels will include growth analysis, percent kernels aborted, measurement of sugars and amino acids, measurement of activities of key enzymes of carbohydrate and protein metabolism, and quantification of steady-state mRNA levels encoding for key enzymes and storage proteins. The remaining kernels will be harvested at maturity (50 DPP) to evaluate effects of heat on kernel dry weight, starch and protein contents. High-temperature tolerant lines will then be compared with high-temperature susceptible lines as to their resistance to *A. flavus* infection and aflatoxin contamination in field trials conducted under heat-stress. Dr. Pratt will guide a breeding program to transfer *A. flavus* resistance into Corn Belt adapted genotypes utilizing RFLP to identify regions of DNA associated with heat-stress tolerance. The inheritance of those marker regions will serve as a guide for selecting progeny of crosses with *A. flavus* resistance.

The Influence of Interaction Between Hybrids and Husk Treatment on Field Aflatoxin Contamination

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Our tests of commercially-grown hybrids have consistently shown that most short-season hybrids are susceptible to preharvest contamination by aflatoxin. This fact has been especially true in the presence of insects. When evaluated with and without insects or inoculation, and at several locations, hybrids with increasing levels of husk tightness sustain lesser amounts of aflatoxin contamination. When husks are manually loosened on hybrids that usually test resistant, the resistance often disappears and the hybrids test susceptible. Those hybrids that do not lose resistance when husks are loosened, give evidence of having a chemical basis for their resistance to aflatoxin accumulation.

Aflatoxin Workshop - October 21-22, 1991
N. Zummo and G.E. Scott, USDA-ARS

<u>Biocompetition to Eliminate Aflatoxin from Corn</u>: *Fusarium moniliforme* is frequently recovered from symptomless maize kernels from ears inoculated in the field with *Aspergillus flavus* in Mississippi. When maize ears were inoculated simultaneously with *F. moniliforme* and *A. flavus* and with *A. flavus* alone in 1990, significantly fewer kernels (25%) were infected with *A. flavus* than kernels from ears inoculated with *A. flavus* alone. These kernels contained significantly less (53%) aflatoxin than kernels from ears inoculated with *A. flavus* alone in two tests in 1990. The results from these tests suggest that *F. moniliforme* can inhibit kernel infection by *A. flavus* in inoculated maize ears and lead to reduced aflatoxin contamination in these kernels.

<u>Progress in Breeding for Resistance to Kernel Infection by *A. flavus* in Corn</u>: A reliable method for inoculating corn ears with *Aspergillus flavus* that does not injure kernels has been developed at Starkville, Mississippi. This inoculation technique may also be used to compare *Aspergillus* isolates or species with each other or with other fungi on uninjured corn kernels. Corn genotypes with resistance to kernel infection by *A. flavus* have been identified using this inoculation method.

The availability of new evaluation methodology and germplasm resistant to kernel infection by *A. flavus* will allow commercial corn breeders to incorporate this resistance into commercial corn hybrids for use by corn farmers. This in turn will reduce the amount of corn grain in the United States that is prohibited from interstate commerce due to aflatoxin contamination.

Two corn genotypes (Mp313E and Mp420) have been identified at Starkville, Mississippi as sources of resistance to kernel infection by *A. flavus* and released to the public. These two inbred lines are currently the only known sources of resistance to the aflatoxin producing fungus *A. flavus*.

The identification and release of these two inbred lines with resistance to kernel infection by *A. flavus* will provide germplasm for breeding programs needed to develop hybrids for use in areas of the country most likely to have an aflatoxin problem. In addition, these genotypes can be used to compare with other untested genotypes to identify additional sources of resistance. We have determined experimentally that corn genotypes that are resistant to kernel infection by *A. flavus* are also resistant to infection by *A. parasiticus*.

<u>Future Plans</u>:

a) We will continue screening corn genotypes for resistance to kernel infection by *A. flavus*.
b) We will continue to search for corn genotypes that when infected by *A. flavus* will have only low levels or no aflatoxin in the grain. We have preliminary data indicating that we have identified a corn genotype with a low level of aflatoxin even when infected by *A. flavus*.
c) We are attempting to develop an even better inoculation method. An inoculation technique that increases kernel infection by *A. flavus* would allow better separation of genotypes for level of resistance. Increased differences for kernel infection by *A. flavus* among genotypes would make genetic tests more feasible and provide opportunities to conduct tests to better understand the host-pathogen interaction.

d) The Vicam Company has developed a rapid method for determining aflatoxin content in grain samples. We are now using this method in the breeding program to select for resistance to aflatoxin contamination in addition to our usual data on kernel infection by the fungus.

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